

TAXONOMIC DISTRIBUTION AND PHYLOGENETIC UTILITY OF GENDER-ASSOCIATED MITOCHONDRIAL GENOMES IN THE UNIONOIDA (BIVALVIA)

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ABSTRACT

Unionoid bivalves exhibit a great diversity in reproductive characteristics. However, the lack of a robust phylogeny severely restricts evolutionary interpretations regarding the genesis and consequences of reproductive character state diversity within the order. The apparent high fidelity of unionoidean doubly uniparental inheritance of mtDNA (DUI), where distinct female- (F) and male- (M) transmitted mtDNA genomes are present, may allow for multiple, independent mtDNA-based estimates of phylogeny and thus contribute to the generation of more robust estimates of unionoid evolutionary history. However, the current lack of knowledge regarding mtDNA transmission patterns in the Etherioidea severely hampers our ability to evaluate the potential of DUI for explicating unionoid phylogeny. This situation prompted us to address the following questions in this study: (1) Is DUI found in the Etherioidea? (2) What is the relative phylogenetic utility of F, M, and concatenated F + M cytochrome *c* oxidase subunit I (*cox1*) sequences for elucidating higher level unionoid evolutionary relationships? (3) What can trees derived from F and M sequence analyses tell us about the evolution of unionoid DUI and other reproductive characters?

Forty-seven species representing all six families within the Unionoida were evaluated, using PCR-based methods, for the presence of DUI. Phylogenetic analyses were carried out on unionoid species for which complementary F and M *cox1* DNA sequences were available as well as on a much more taxonomically inclusive F *cox1* data set. We determined that (1) the Etherioidea likely lacks DUI; (2) M and F + M *cox1*-based analyses provide better resolved estimates of unionoidean relationships than do F *cox1*-based analyses; and (3) the F and M non-concatenated *cox1* inclusive phylogenetic analyses suggest the inference that (a) the presence of DUI, glochidial larvae, and endobranched brooding are the ancestral unionoid character states, (b) both DUI and glochidial larvae were lost in the ancestral etherioidean lineage, (c) margaritiferids are closely related to unionids and exhibit a derived suite of morphological characteristics, and (d) a clarification of the evolutionary dynamics of unionoid DUI and other reproductive characteristics will require a robust phylogeny for the order that is based on multiple data sets.

Key words: DUI, *cox1*, mtDNA, Hyriidae, Margaritiferidae, Unionidae, Iridinidae, Mycetopodidae.

INTRODUCTION

Freshwater unionoid bivalves exhibit significant taxonomic diversity (~175 genera) and a broad geographic distribution that includes all continents, with the exception of Antarctica (Simpson, 1896, 1900, 1914; Haas, 1969; Starobogatov, 1970). Following Parodiz & Bonetto (1963), the bivalve order Unionoida is comprised

of six families contained within two superfamilies of freshwater mussels (Superfamily Etherioidea: Etheriidae, Iridinidae and Mycetopodidae; Superfamily Unionoidea: Hyriidae, Margaritiferidae, and Unionidae). However, the concept of the Etheriidae as a monophyletic group containing all cemented unionoid bivalves has been rejected (Bogan & Hoeh, 2000), thus its usage herein is applied only to the genus *Etheria*.

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Unionoid bivalves also exhibit great diversity, among higher taxa, in their reproductive characteristics, such as the morphology of their parasitic larvae as well as the larval brooding location. There are two types of parasitic unionoid larvae: the bivalved glochidium (Hyriidae, Margaritiferidae, and Unionidae) and the univalved lasidium (Iridinidae and Mycetopodidae) (Wächtler et al., 2001). It has been suggested that the extreme morphological divergence between these two types of larvae indicates that the unionoidean and etherioidean bivalves represent independently

derived freshwater lineages and thus the Unionoida is a polyphyletic assemblage (Parodiz & Bonetto, 1963). However, the results of recent unionoid phylogenetic analyses reject the latter hypothesis (e.g., Hoeh et al., 2001; Roe & Hoeh, 2003). In addition to the extreme distinctions in larval morphology, unionoid higher taxa also exhibit differences in larval brooding location. Unionoids use three general brooding locations. Tetragenous brooders utilize all four ctenidia as marsupia (Margaritiferidae and some Unionidae), endobranchous brooders utilize only the inner two ctenidia

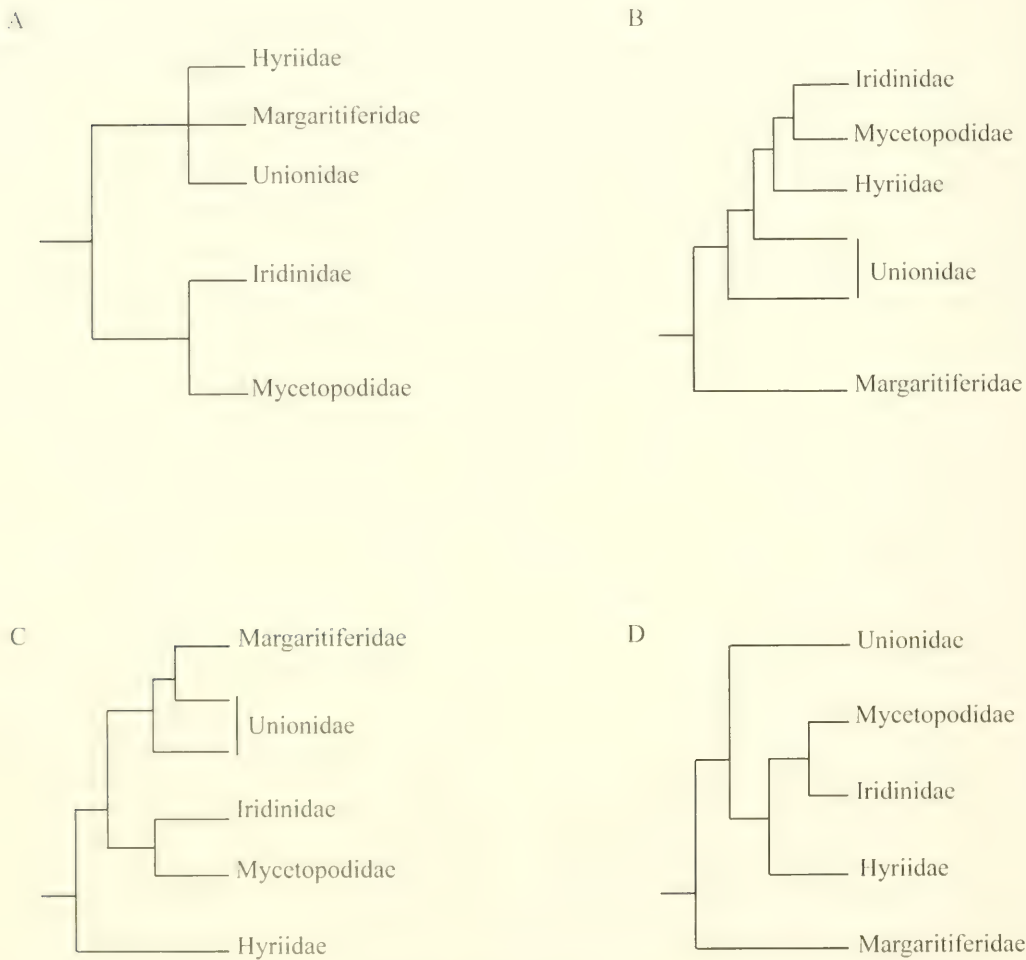


FIG. 1. Simplified representations of unionoid familial relationships based on the A: Classifications of Simpson (1900) and Parodiz & Bonetto (1963); B: Phylogenetic analyses of morphological characters after Graf (2000) and Hoeh et al. (2001); C: Phylogenetic analysis of combined morphological and molecular characters after Hoeh et al. (2001); D: Phylogenetic analysis of morphological and molecular characters after Roe & Hoeh (2003).

(Hyriidae, Iridinidae, and Mycetopodidae), and ectobranchous brooders utilize only the outer two ctenidia (some Unionidae). Increasing our understanding regarding the evolution of this diversity in reproductive structures has been a major, if largely unrealized, goal of recent unionoid phylogenetic studies (e.g., Graf & Ó Foighil, 2000; Hoeh et al., 1998a, 2001).

Numerous hypotheses of unionoid evolutionary relationships, based on analyses of both morphological and DNA characters, have been published recently (Bogan & Hoeh, 2000; Graf & Ó Foighil, 2000; Hoeh et al., 1998a, 2001; Graf, 2000; Roe & Hoeh, 2003). Despite this flurry of recent studies, when comparing hypotheses of unionoid familial relationships, it becomes apparent that there is little agreement (Fig. 1). Parodiz & Bonetto's (1963) classification mirrors that of Simpson (1896, 1900), in describing Mycetopodidae + Iridinidae (Simpson's Mutelidae) as fundamentally distinct from the Unionidae + Margaritiferidae + Hyriidae (Simpson's Unionidae) (Fig. 1A). Recent phylogenetic analyses offer neither corroboration of this view nor significant among-analysis congruence. Analyses of morphological characters presented by Graf (2000) and Hoeh et al. (2001) both return the Margaritiferidae as the basal unionoid lineage, a paraphyletic Unionidae, and the Hyriidae + Mycetopodidae + Iridinidae as a derived lineage (Fig. 1B). In contrast, both the molecular (i.e., *cox1* DNA sequences) and the combined analysis of morphological and molecular data presented by Hoeh et al. (1998a, 2001) (Fig. 1C) place the Hyriidae as the basal unionoid lineage, with the Unionidae returned as paraphyletic. Yet another combined analysis of morphological and molecular (i.e., *cox1* DNA sequences) characters (Roe & Hoeh, 2003; Fig. 1D), this time using binary coding of the morphological data and a posteriori character weighting, returns the Margaritiferidae as basal and a monophyletic Unionidae as sister to a Hyriidae + Iridinidae + Mycetopodidae clade. As is readily apparent from the above comparisons, ambiguity still remains when attempting to explain the evolution of unionoid diversity. This difficulty results from the fact that the only degree of stability exhibited across all of the relationship hypotheses above is that the Mycetopodidae and Iridinidae are always returned as closely related. Importantly, the topological positions of the Hyriidae and Margaritiferidae do not remain stable across analyses and thus, we are left

with the lack of a well-resolved phylogeny for the Unionoida. This situation severely restricts evolutionary interpretations regarding the genesis and consequences of reproductive character state diversity within the order.

A largely underutilized set of phylogenetically informative characters exists in the male-transmitted mtDNA genomes within the Unionoidea, as a consequence of the presence of doubly uniparental inheritance of mtDNA (DUI) in that taxon. DUI has been observed in two orders of marine bivalves (Mytiloida: Skibinski et al., 1994; Zouros et al., 1994; Hoeh et al., 1996; and Veneroida: Passamonti & Scali, 2001) and the freshwater bivalve superfamily Unionoidea (Hoeh et al., 1996; Liu et al., 1996). In species with this type of mtDNA inheritance, there are distinct female-(F) and male-(M) transmitted genomes. Typically, females are homoplasmic for the F genome, whereas males are heteroplasmic, that is, they contain both the F and M genomes (Skibinski et al., 1994; Zouros et al., 1994). In males, these two distinct mtDNA genomes segregate by tissue type. The F genome predominates in somatic tissues while the M genome is concentrated in spermatogenic tissues (Stewart et al., 1995; Garrido-Ramos et al., 1998). Therefore, taxa possessing DUI transmit two distinct mtDNA genomes. Females pass on their F genome to both male and female progeny while males transmit their M genome only to male progeny. While earlier mtDNA-based analyses of unionoid phylogeny largely made use of F genome sequences (e.g., Graf & O' Foighil, 2000; Hoeh et al., 1998a, 2001), more recent phylogenetic analyses of both F and M genome DNA sequences, from exemplar species representing the Hyriidae, Margaritiferidae, and Unionidae, have produced evolutionary trees with distinct F and M clades exhibiting very similar topologies (Curolle & Kocher, 2002, 2005; Hoeh et al., 2002). The observed reciprocal monophyly of these topologically similar F and M clades, in conjunction with fossil evidence, suggests that DUI has been operating at a high level of fidelity in the Unionoidea for more than 100 my (Curolle & Kocher, 2002, 2005; Hoeh et al., 1996, 2002).

The fidelity of the DUI system is sometimes compromised in mytiloids. Evidence supporting this view comes from phylogenetic analyses (e.g., Hoeh et al., 1997) and breeding studies (Fisher & Skibinski 1990; Zouros et al., 1994; Rawson et al., 1996; Saavedra et

al., 1997; Quesada et al., 1999; Ladoukakis et al., 2002). For example, taxonomically more inclusive phylogenetic analyses of F and M genomes in mytiloids have failed to recover distinct F and M clades (e.g., Hoeh et al., 1996, 1997). As one example, some *Mytilus* M genomes appear more closely related to F genomes than to other similarly transmitted genomes (Hoeh et al. 1997). Failure to inherit the M genome may result in recruitment and masculinization of the F genome to function as a newly derived "M" genome (Hoeh et al., 1996, 1997). Initially after masculinization, the F and the newly derived "M" genome have identical DNA sequences. Subsequently, divergence between the F and "M" genome begins *de novo* (Hoeh et al., 1996, 1997). Additionally, mytiloid masculinization events have been observed in laboratory crosses (Zouros et al., 1994; Saavedra et al., 1997) as well as in natural populations (Fisher & Skibinski, 1990; Rawson et al., 1996; Quesada et al., 1999; Ladoukakis et al., 2002). This presents a problem when using F and M genomes in a complementary manner for mytiloid phylogenetic analyses as non-orthologous comparisons could result. To date, feminization, or recruitment of an M genome to function as the F, has not been observed or inferred for any taxa with DUI. Unlike the situation in mytiloids, masculinization events have not been documented for the Unionoidea (Hoeh et al., 1996, 2002; Curolé & Kocher, 2002, 2005). This apparent high fidelity of unionoidan DUI may allow for multiple, independent mtDNA-based estimates of phylogeny and genetic variation within the order (Hoeh et al., 2002; Krebs, 2004).

The presence of DUI in the hyriid, margaritifera, and unionid specimens sampled to date prompted us to address the following questions in this study: (1) Is DUI found in the Etheriidae, Iridinidae and Mycetopodidae? If so, DUI likely represents the ancestral mtDNA transmission pattern for unionoid bivalves. If not, DUI presence/absence data may be informative regarding unionoid familial relationships. (2) What is the relative phylogenetic utility of F, M, and concatenated F + M cytochrome c oxidase subunit I (*cox1*) sequences for elucidating higher level unionoid evolutionary relationships? (3) What can trees derived from F and M sequence analyses tell us about the evolution of unionoid DUI and reproductive characters?

MATERIALS AND METHODS

Taxa sampled in this study for the presence of a male genome included 47 species representing all six families within the Unionoidea (Table 1). Gender was determined by microscopical examination of gonadal tissues. Total genomic DNA was isolated from either somatic (mantle or foot) or testis tissue using the Qiagen DNeasy animal kit. An approximately 710 bp fragment of *cox1* was amplified from both the F and M mtDNA genomes using modified versions of the universal *cox1* primers (Folmer et al., 1994): LCO22me2 5'-GGTCAACAAAYCATAARGATATTGG-3'; HCO700dy2, 5'-TCAGGGTGACCAAAAAAYCA-3'. To efficiently screen for the presence of the M genome, largely gender-specific *cox2* primers were used to amplify the *cox2-cox1* fragment used by Curolé & Kocher (2002). These primers were chosen due to the size difference exhibited between the F and M *cox2-cox1* fragments as described by Curolé & Kocher (2002). The "male-specific" *cox2* primer was UNIOCOII.2 (Curolé, 2004) and a "female-specific" primer (UNIOCOII.2b, 5'-CAGTGRTATTGRRVDTAYGA-3') was derived from the UNIOCOII.2 primer and other unionid F sequences available from GenBank. Both "gender-specific" primers were paired with HCO700dy2 to amplify the *cox2-cox1* fragment. These primers typically amplified approximately 1.1 Kbp of *cox2-cox1* from F genomes and approximately 1.7 Kbp from M genomes. PCR reactions consisted of 1X Qiagen PCR buffer, 0.2 mM each dNTP, 0.5 μ M each primer, and Qiagen *Taq*. Reactions using the *cox1* primer pair were cycled at 94°C for 60 s, 40°C for 60 s, and 72°C for 60 s for a total of 40 cycles and reactions using the male-specific *cox2* primer were cycled at 94°C for 60 s, 50°C for 60 s, and 72°C for 120 s for a total of 40 cycles. Reactions involving the female-specific *cox2* primer followed the same profile given above for the male specific primer but were annealed at 46°C. Sequencing template purification was carried out following Folmer et al. (1994). The *cox1* fragment yielded 619 bp of sequence via cycle sequencing with Perkin Elmer AmpliCycle Sequencing Kits using ddNTP-dNTP ratios optimized for automated sequencing. Sequences were obtained from both strands of the *cox1* fragment and the dye-labeled *cox1* sequencing primers were of the same sequence as the PCR prim-

TABLE 1. Taxa evaluated for the presence/absence of F and M *cox2-cox1* amplicons; + = amplification successful, - = amplification failed, NA = amplification not attempted.

Family	Species	<i>cox2-cox1</i> amplicon F	<i>cox2-cox1</i> amplicon M
Unionidae	<i>Actinonaias ligamentina</i>	+	+
	<i>Amblema plicata</i>	+	+
	<i>Anodonta californiensis</i>	+	+
	<i>Cyprogenia aberti</i>	+	+
	<i>Cyrtonaias tampicoensis</i>	-	+
	<i>Dromus dromas</i>	+	+
	<i>Ellipsaria lineolata</i>	+	+
	<i>Elliptio dilatata</i>	+	+
	<i>Epioblasma brevidens</i>	+	+
	<i>Fusconaia flava</i>	+	+
	<i>Glebula rotundata</i>	+	+
	<i>Hamiota subrotundata</i>	+	+
	<i>Lampsilis cardium</i>	+	+
	<i>Lampsilis hydiana</i>	+	+
	<i>Lampsilis powellii</i>	+	+
	<i>Lampsilis reeveiana</i>	+	+
	<i>Lampsilis siliquioidea</i>	+	+
	<i>Lampsilis straminea</i>	+	+
	<i>Lampsilis streckeri</i>	+	+
	<i>Lampsilis teres</i>	+	+
	<i>Leptodea fragilis</i>	+	+
	<i>Leptodea leptodon</i>	+	+
	<i>Ligumia recta</i>	+	+
	<i>Medionidus conradicus</i>	+	+
	<i>Obovaria olivaria</i>	+	+
	<i>Popenaias popeii</i>	+	+
	<i>Potamilus alatus</i>	+	+
	<i>Potamilus capax</i>	+	+
	<i>Potamilus ohioensis</i>	+	+
	<i>Potamilus purpuratus</i>	+	+
	<i>Ptychobranthus fasciolaris</i>	+	+
	<i>Toxolasma glans</i>	+	+
	<i>Truncilla truncata</i>	+	+
	<i>Venustaconcha ellipsiformis</i>	+	+
	<i>Villosa iris</i>	+	-
	<i>Villosa lienosa</i>	+	+
	<i>Villosa villosa</i>	+	+
Margaritiferidae	<i>Cumberlandia monodonta</i>	+	+
	<i>Dahurinaia</i> sp.	-	+
	<i>Margaritifera margaritifera</i>	-	+
Hyriidae	<i>Hyridella menziesi</i>	+	+
Iridinidae	<i>Chambardia rubens</i>	+	-
	<i>Mutela dubia</i>	+	-
Etheriidae	<i>Etheria elliptica</i>	+	-
Mycetopodidae	<i>Anodontites guanarensis</i>	+	-
	<i>Tamsiella tamsiana</i>	+	-
Neotrigoniidae	<i>Neotrigonia margaritacea</i>	-	NA

ers. The 3' portion of the M *cox2-cox1* fragment was sometimes sequenced, using the HCO700dy2 sequencing primer, to confirm M *cox1* sequences generated by the *cox1* primer pair. Sequences were visualized using Li-Cor 4200L-2 and 4200S-2 DNA sequencers and initial base calls were made by e-Seq v 2.0. Contiguous sequences were assembled and verified using AlignIR v2.0 and final sequence alignments were completed manually with MacClade v4.0. GenBank accession numbers for the *cox1* sequences generated and/or analyzed herein are given in Table 2. All testis extraction-derived *cox1* sequences were added to a matrix containing confirmed F and M *cox1* sequences and phylogenetic analyses were used to test the putative M status of the newly generated sequences. Subsequent to the initial attempts to amplify the M *cox2-cox1* fragment from their testis-derived total DNAs, multiple attempts were made to amplify an M mitochondrial fragment from members of the Iridinidae and Mycetopodidae using two, intragenic universal primer pairs (i.e., *cox1* and 16S [LR-J-12887, LR-N-13398; Simon et al., 1994]).

Three complementary F and M sequence data sets, populated by the unionoid species from which both F and M *cox1* sequences were obtained, were analyzed using the maximum likelihood (ML) and maximum parsimony (MP) algorithms contained in PAUP* (v.4.0b10; Swofford, 2001). Bayesian inference (BI) analyses were carried out with MrBayes v3.0b4 (Huelsenbeck & Ronquist, 2003). The complementary F and M genome *cox1* sequences were analyzed both individually and in an F + M concatenated manner. Recent literature indicates that a total evidence approach can produce the best tree topologies (e.g., Collin, 2003; Creer et al., 2003; Hassanin & Douzery, 2003; Schwarz et al., 2003). Thus, the F + M *cox1* concatenated trees were used as the best estimates of the phylogenetic relationships among the unionoid sequences examined. Additional phylogenetic analyses were carried out, using the BI and MP algorithms, on an inclusive non-concatenated *cox1* DNA sequence data set that contained a much broader taxonomic sampling of the available F *cox1* sequences as well as all available M *cox1* sequences. Modeltest (v. 3.6; Posada & Crandall, 1998) was used to determine which model best fit the F, M, and F + M sequence data. The GTR + G + I model was used in all BI and ML analyses. *Neotrigonia margaritacea*

(Trigonioida) *cox1* sequences were used to root the trees derived from the complementary data set analyses (e.g., Hoeh et al., 1998a), while a much broader sampling of taxa was used to root the trees derived from analyses of the inclusive data set (e.g., Hoeh et al., 2002).

A total of 29 *cox1* sequences were included in the complementary data set phylogenetic analyses while 105 sequences were present in the inclusive non-concatenated M and F data set. Each of the four BI analyses consisted of 10 chains, 5 million generations, and a 2 million generation burn-in. PAUP* was used to select, from among all of the 1,000 saved BI trees from each of the complementary data set analyses, the topologies with the highest log likelihood scores. Due to the saturation of third position transitions (e.g., Hoeh et al., 1998a), all MP analyses were conducted on transformed *cox1* sequences such that third position transitions were excluded from analyses. Multiple random terminal taxa addition sequence runs, combined with global branch rearrangement options, were employed when generating topologies, from the complementary data sets, via the ML and MP algorithms. These options increased the probability of finding the actual best topology under each of these two optimality criteria (e.g., Hendy et al., 1988; Maddison, 1991). Standard non-parametric bootstrap (Felsenstein, 1985) analyses were carried out to evaluate the level of support for particular nodes obtained from the ML (1,000 bootstrap replicates) and MP (10,000 bootstrap replicates; 100,000 fast-heuristic replicates for the inclusive data set) analyses. A parsimony-based ILD test (Farris, 1994), as implemented in PAUP*, was used to test for incongruence between the F and M *cox1* sequences.

RESULTS AND DISCUSSION

What is the Taxonomic Distribution of DUI within the Unionoida?

Definitively M genome *cox2-cox1* fragments were amplified from 40 species representing three (Hyriidae, Margaritiferidae, and Unionidae) of the six unionoid families (Table 1). Sequences from *cox1* confirmed that the long *cox2-cox1* PCR fragments obtained from testis-based DNA extractions were from M genomes. However, testis-based DNA extrac-

TABLE 2. Source taxa and GenBank accession numbers for the *cox1* DNA sequences used in phylogenetic analyses.

Family	Species	GenBank Accession No.	
		F	M
Unionidae	<i>Actinonias ligamentina</i>	AF231730	AF406796
	<i>Cyrtonaias tampicoensis</i>	AF231749	AF406798
	<i>Fusconaia flava</i>	AF231733	AF406799
	<i>Gonidea angulata</i>	DQ206792	DQ206794
	<i>Lampsilis teres</i>	AF406803	AF406794
	<i>Ligumia recta</i>	AF231748	AF406795
	<i>Potamilus purpuratus</i>	AF406804	AF406797
	<i>Pseudodon vondembuschianus</i>	DQ206793	DQ206795
	<i>Pyganodon fragilis</i>	AF406805	AF406800
	<i>Pyganodon grandis</i>	AF231734	AF406801
Margaritiferidae	<i>Cumberlandia monodonta</i> 1	AY785393	AY785397
	<i>Cumberlandia monodonta</i> 2	AF156498	
	<i>Cumberlandia monodonta</i> 3	AF156497	
	<i>Cumberlandia monodonta</i> 4	AY579131	
	<i>Dahurinaia dahurica</i> 1	AY579123	
	<i>Dahurinaia dahurica</i> 2		AY785400
	<i>Dahurinaia dahurica</i> 3		DQ241802
	<i>Margaritifera auricularia</i> 1	AY579125	
	<i>Margaritifera auricularia</i> 2	AF303312	
	<i>Margaritifera auricularia</i> 3	AF303313	
	<i>Margaritifera auricularia</i> 4	AF303315	
	<i>Margaritifera falcata</i> 1	AY579126	
	<i>Margaritifera falcata</i> 2	AY579128	
	<i>Margaritifera falcata</i> 3	AY579127	
	<i>Margaritifera laevis</i>	AY579124	
	<i>Margaritifera margaritifera</i> 1	AF303319	
	<i>Margaritifera margaritifera</i> 2	AF303320	
	<i>Margaritifera margaritifera</i> 3	AF303336	
	<i>Margaritifera margaritifera</i> 4	AF303341	
	<i>Margaritifera margaritifera</i> 5	AY579129	
	<i>Margaritifera margaritifera</i> 6	AY579130	
	<i>Margaritifera margaritifera</i> 7	AF303331	
	<i>Margaritifera margaritifera</i> 8	AF303332	
	<i>Margaritifera margaritifera</i> 9	AF303338	
	<i>Margaritifera margaritifera</i> 10	AF303340	
	<i>Margaritifera margaritifera</i> 11	AF303335	
	<i>Margaritifera margaritifera</i> 12	AF303337	
	<i>Margaritifera margaritifera</i> 13	U56847	
	<i>Margaritifera margaritifera</i> 14	DQ060171	
	<i>Margaritifera margaritifera</i> 15	AF303339	
	<i>Margaritifera margaritifera</i> 16		AY785399
	<i>Margaritifera margaritifera durrovensis</i> 1	AF303344	
	<i>Margaritifera margaritifera durrovensis</i> 2	AF303345	
	<i>Margaritifera margaritifera durrovensis</i> 3	AF303346	
	<i>Margaritifera margaritifera durrovensis</i> 4	AF303347	
	<i>Margaritifera margaritifera durrovensis</i> 5	AF303342	
	<i>Margaritifera margaritifera durrovensis</i> 6	AF303343	

(continues)

(continued)

Family	Species	GenBank Accession No.	
		F	M
Hyriidae	<i>Alathyria jacksoni</i> 1	AY386977	
	<i>Alathyria jacksoni</i> 2	AY386981	
	<i>Alathyria jacksoni</i> 3	AY386970	
	<i>Alathyria jacksoni</i> 4	AY386974	
	<i>Castalia stevensi</i>	AF231736	
	<i>Diplodon deceptus</i>	AF231736	
	<i>Hyridella australis</i>	AF305367	
	<i>Hyridella depressa</i> 1	AF156496	
	<i>Hyridella depressa</i> 2	AF305368	
	<i>Hyridella menziesi</i> 1	AF231747	
	<i>Hyridella menziesi</i> 2		AF406802
	<i>Lortiella rugata</i>	AF231746	
	<i>Velesunio ambiguus</i> 1	AF305371	
	<i>Velesunio ambiguus</i> 2	AF305372	
	<i>Velesunio ambiguus</i> 3	AY211582	
	<i>Velesunio ambiguus</i> 4	AY211586	
	<i>Velesunio angasi</i>	AF231743	
	<i>Velesunio</i> sp. 1	AY387018	
	<i>Velesunio</i> sp. 2	AY386999	
	<i>Velesunio</i> sp. A 1	AY211550	
	<i>Velesunio</i> sp. A 2	AY211554	
	<i>Velesunio</i> sp. B 1	AY211558	
	<i>Velesunio</i> sp. B 2	AY211566	
	<i>Velesunio</i> sp. D 1	AY211587	
	<i>Velesunio</i> sp. D 2	AY211598	
Iridinidae	<i>Chambardia rubens</i> 1	DQ241807	
	<i>Chambardia rubens</i> 2	DQ241808	
	<i>Chambardia rubens</i> 3	AY785389	
	<i>Mutela dubia</i> 1	DQ241805	
	<i>Mutela dubia</i> 2	AY785388	
	<i>Mutela dubia</i> 3	DQ241806	
	<i>Mutela rostrata</i> 1	AY785387	
	<i>Mutela rostrata</i> 2	DQ241804	
Mycetopodidae	<i>Acostaea rivolii</i>	AF231739	
	<i>Anodontites guaranensis</i>	AY785383	
	<i>Anodontites trigonus</i>	AF231738	
	<i>Monocondylaea minuana</i>	AF231745	
	<i>Tamsiella tamsiana</i>	AY785384	
Etheriidae	<i>Etheria elliptica</i> 1	DQ241803	
	<i>Etheria elliptica</i> 2	AF231739	
Outgroup taxa	<i>Albinaria turrita</i>	X71393	
	<i>Dentalium</i> sp.	U56843	
	<i>Drosophila yakuba</i>	X03240	
	<i>Katharina</i> sp.	U56845	
	<i>Lepetodrilus elevatus</i>	U56846	
	<i>Neotrigonia margaritacea</i>	U56850	
	<i>Solemya velum</i>	U56852	

tions from all five species representing the Etheriidae, Iridinidae, and Mycetopodidae failed to yield the expected long M *cox2-cox1* fragment. Regarding the total DNAs extracted from representatives of these three families, all PCR attempts resulted in amplification of an F genome fragment from both mantle and testis DNA extractions. Subsequent sequencing and phylogenetic analyses of these fragments confirmed that identical sequences (all from F genomes) had been amplified from both mantle and testis extractions from the same individuals. Furthermore, the *cox1* and 16S intragenic primer pairs failed to produce an M genome fragment. This corroborates the failure of the intergenic *cox2-cox1* primers to amplify an M fragment from the sampled etherioidean individuals.

The Etheriidae, Iridinidae, and Mycetopodidae represent closely related taxa, and have typically been given distinct superfamilial status, Etherioidea (Parodiz & Bonetto, 1963; Hoeh et al., 1998b, 2001; Bogan & Hoeh, 2000; Roe & Hoeh, 2003). Given their phylogenetic propinquity, it is not surprising that representatives of these three families would produce similar, yet unexpected, results: failure to yield M genome amplicons. There appear to be three possible explanations for the failure of representatives of the Etheriidae, Iridinidae and Mycetopodidae to yield M fragments. (1) Recent masculinization events have occurred such that the newly recruited "M genomes" (originally F genomes) are amplified. (2) The primers failed to anneal to the M sequence due to the rapidly evolving nature of the M genomes (Rawson & Hilbish, 1995; Stewart et al., 1995; Curole & Kocher, 2002; Hoeh et al., 2002; Krebs, 2004). (3) These taxa do not possess DUI.

Mitochondrial DNA masculinization events in DUI-containing taxa were first postulated for, and later supported with data from, *Mytilus* by Hoeh et al. (1996, 1997). Subsequently, other investigators have corroborated the existence of the mtDNA masculinization process in mytiloid but not in unionoid bivalves (e.g., Zouros et al., 1994; Hoeh et al., 1996, 2002; Saavedra et al., 1997). However, if the masculinization hypothesis is to be invoked as the explanation for our observations, we would expect to observe distinct etherioidean M mtDNA sequences that are more closely related to F sequences than to other M sequences. Our repeated observations of identical mtDNA sequences from testis- and

mantle-derived DNA extractions from each etherioidean individual examined do not meet these expectations. Immediately after a masculinization event, it is predicted that the F and new "M" (i.e., recently converted from the female- to the male-transmission route) genomes will be identical. However, under the masculinization hypothesis, it is extremely unlikely that we would have observed identical *cox1* sequences from separate mantle and testis DNA extractions from individuals representing five species as this would require multiple independent, approximately simultaneous, and relatively recent masculinization events.

The M genomes in both unionoid and mytiloid bivalves have a significantly greater rate of substitution relative to that estimated for the corresponding F genomes (Skibinski et al., 1994; Hoeh et al., 1996, 2002; Liu et al., 1996; Stewart et al., 1996; Quesada et al., 1998; Krebs, 2004). This may be due to a higher mutation rate for the M genomes, smaller effective population size for the M genomes, positive selection for the M genomes, relaxed selection for the M genomes, or a combination of these processes (Stewart et al., 1996; Passamonti et al., 2003). Nevertheless, an elevated rate of substitution has been suggested as the explanation for the occasional failure of universal mtDNA primer pairs to amplify M genomes (Rawson & Hilbish, 1995; Stewart et al., 1995; Curole & Kocher, 2002; Hoeh et al., 2002; Krebs, 2004). Our use of three distinct, conserved primer pairs to attempt amplification of etherioidean M genomes, with failure to do so in each instance, suggests that either (1) all of the sampled etherioidean specimens have very divergent M mtDNA sequences for 16S, *cox1*, and *cox2* or (2) these specimens lack DUI. We believe that multiple failed M-fragment amplification attempts, using both intra- and inter-genic conserved primer pairs, render the former hypothesis unlikely.

We thus believe that it is likely that DUI is absent from the Etherioidea. This leads to the question of whether the absence of DUI in this superfamily indicates a loss in the ancestral etherioidean lineage or a gain of DUI in the ancestral unionoid lineage (after Parodiz & Bonetto, 1963). Unfortunately, due to the lack of a robust unionoid phylogeny and information regarding the presence/absence of DUI in *Neotrigonia*, there remains no *a priori* way to rigorously evaluate which condition is apo-

morphic and thus, the phylogenetically informative character state. If DUI was derived within the Unionoida, then it may represent an apomorphy for the Unionoidea, whereas a loss of DUI in the common etherioidean ancestor would represent an apomorphy for the etherioids and the presence of DUI would represent a plesiomorphy for the Unionoida. Robust inferences regarding the evolutionary dynamics of unionoid DUI depend upon the existence of a robust phylogeny for the Unionoida. As mentioned previously, phylogenetic analyses to date, utilizing partial *F cox1* sequences (Folmer fragment), have been unable to robustly resolve unionoid familial relationships. However, *M cox1* sequences have demonstrated the ability to increase topological resolution when analyzed alone or in conjunction with *F cox1* sequences (Hoeh et al., 2002). Utilization of the relatively new model-based Bayesian phylogenetic methods appears to reveal additional phylogenetic signal contained within existing *F cox1* sequences.

What Can Analyses of Complementary F and M *cox1* Sequences Tell us About Higher Level Unionoidean Relationships?

Failure to amplify M genome fragments from any etherioid taxa obviously prevents us from conducting any taxonomically inclusive M genome-based higher level phylogenetic analyses of unionoid relationships. Given this serious limitation, what can analyses of complementary F and M *cox1* sequence data sets tell us about higher level unionoidean (*sensu* Parodiz & Bonetto, 1963) relationships? Phylogenetic analyses of *F cox1* sequences recover *Hyridella* as the basal unionoidean lineage; however, the Unionidae are not recovered as monophyletic (Fig. 2). Specifically, *Cumberlandia*, a margaritiferid, is depicted as the sister taxon to *Fusconaia* and this placement renders the Unionidae paraphyletic. Nodal support levels for the interfamilial relationships are relatively low as seen in previously published *F cox1* analyses (e.g., Hoeh et al., 2001). Robust intergeneric nodal support values are only observed for the clade containing the following four lampsiline taxa: *Actinonaias*, *Lampsilis*, *Ligumia*, and *Potamilus*.

Analyses of M *cox1* sequences recovered a robustly supported, monophyletic Unionidae but the relationships among the hyriid, margaritiferid, and unionid taxa were not well resolved (Fig. 3). As in the topology obtained from the *F cox1* analysis, *Hyridella* is repre-

sented as a descendent of the primary unionoidean cladogenic event. In general, the nodal support values derived from analyses of M *cox1* sequences are significantly improved over those of the *F cox1* analysis presented herein (Fig. 2). These results were foreshadowed by previous comparative analyses of F and M sequences (e.g., Hoeh et al., 2002; Krebs, 2004).

Concatenating F and M *cox1* sequences was legitimized by the lack of significant incongruence between the F and M sequences (as indicated by the ILD test, $p = 0.271$). Phylogenetic analyses of the concatenated F and M *cox1* sequences (Fig. 4) produced a topology very similar to that produced by the M *cox1* analyses (Fig. 3). This result was anticipated due to the greater number of parsimony-informative sites in the M *cox1* sequences (Hoeh et al., 2002) and the lack of significant incongruence between the F and M sequences. However, a fundamental difference between the results of the M and F + M analyses is the latter's increased nodal support for margaritiferids (represented herein by *Cumberlandia*) as the sister taxon to the Unionidae. This result strongly supports the basal position of hyriids (represented by *Hyridella*) within the Unionoidea. This basal placement of hyriids is independently supported by Graf's (2002) analyses of 28S sequences, which also lacked representatives of the Etherioidea.

The basal position of *Hyridella* in all of the analyses presented herein is in conflict with the placement of the Margaritiferidae as the basal unionoid lineage as depicted in the morphology-based trees of Graf (2000) and Hoeh et al. (2001), as well as in the total evidence-based tree presented in Roe & Hoeh (2003). However, our topology is consistent with the molecular and total evidence-based topologies of Hoeh et al. (1998b, 2001) as well as with the hypothesis that the relatively "simple" anatomy of margaritiferids is a derived rather than an ancestral condition as often postulated. For example, the loss of ctenidial water tubes in margaritiferids may be the end result of selection for the release of a significantly larger conglutinate mass than that of its ancestor.

If the relative evolutionary relationships postulated from the complementary F and M concatenated *cox1* data set analyses are maintained in subsequent more taxonomically inclusive phylogenetic analyses of the Unionoida, where might a monophyletic Etherioidea attach to our unionoidean tree? Two previously presented hypotheses of unionoid

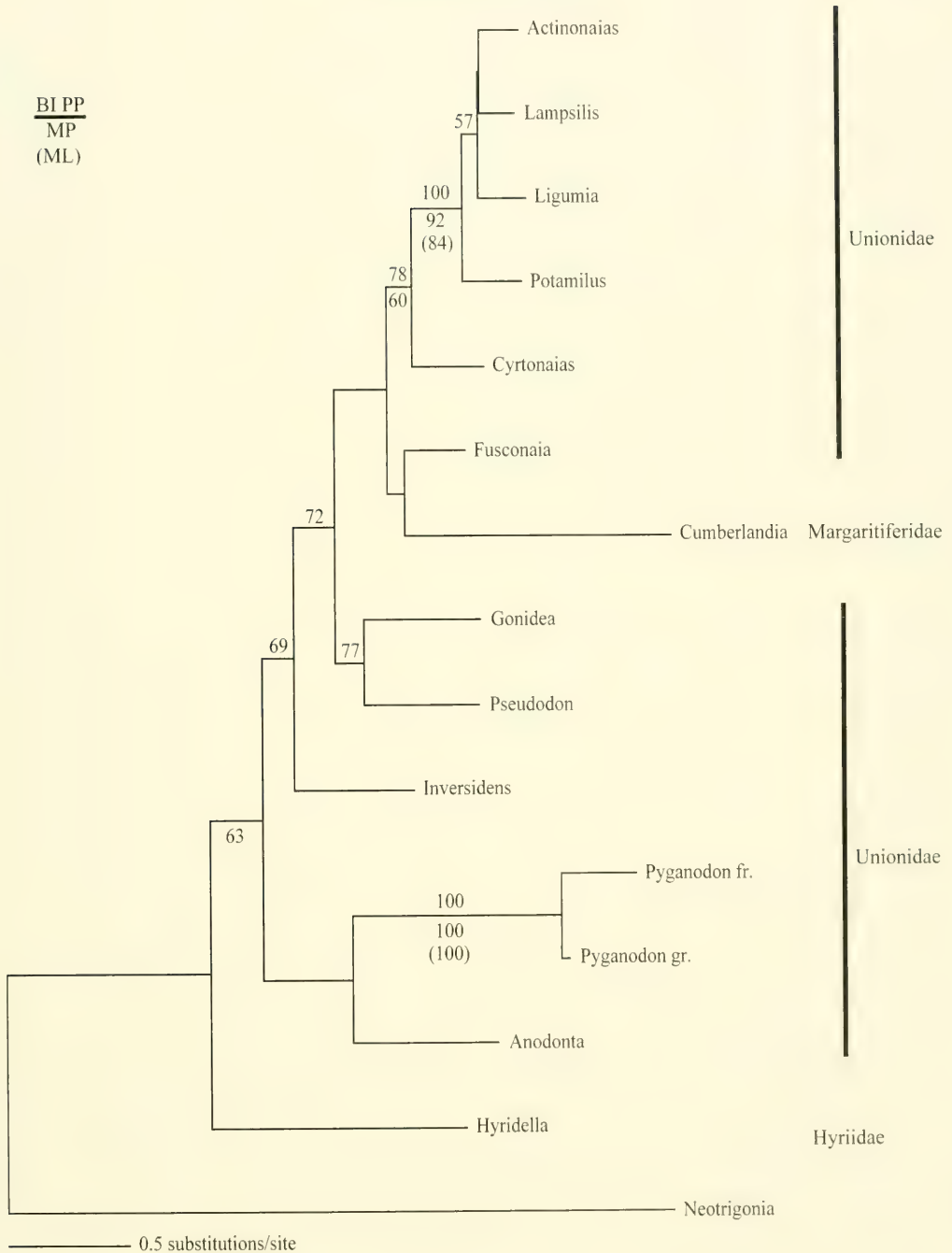


FIG. 2. Best tree found by Bayesian analyses of *F. cox1* sequences (619 bp). When > 50, Bayesian posterior probabilities (x100) presented above internodes, MP bootstrap values and ML bootstrap values (in parentheses) are presented below internodes.

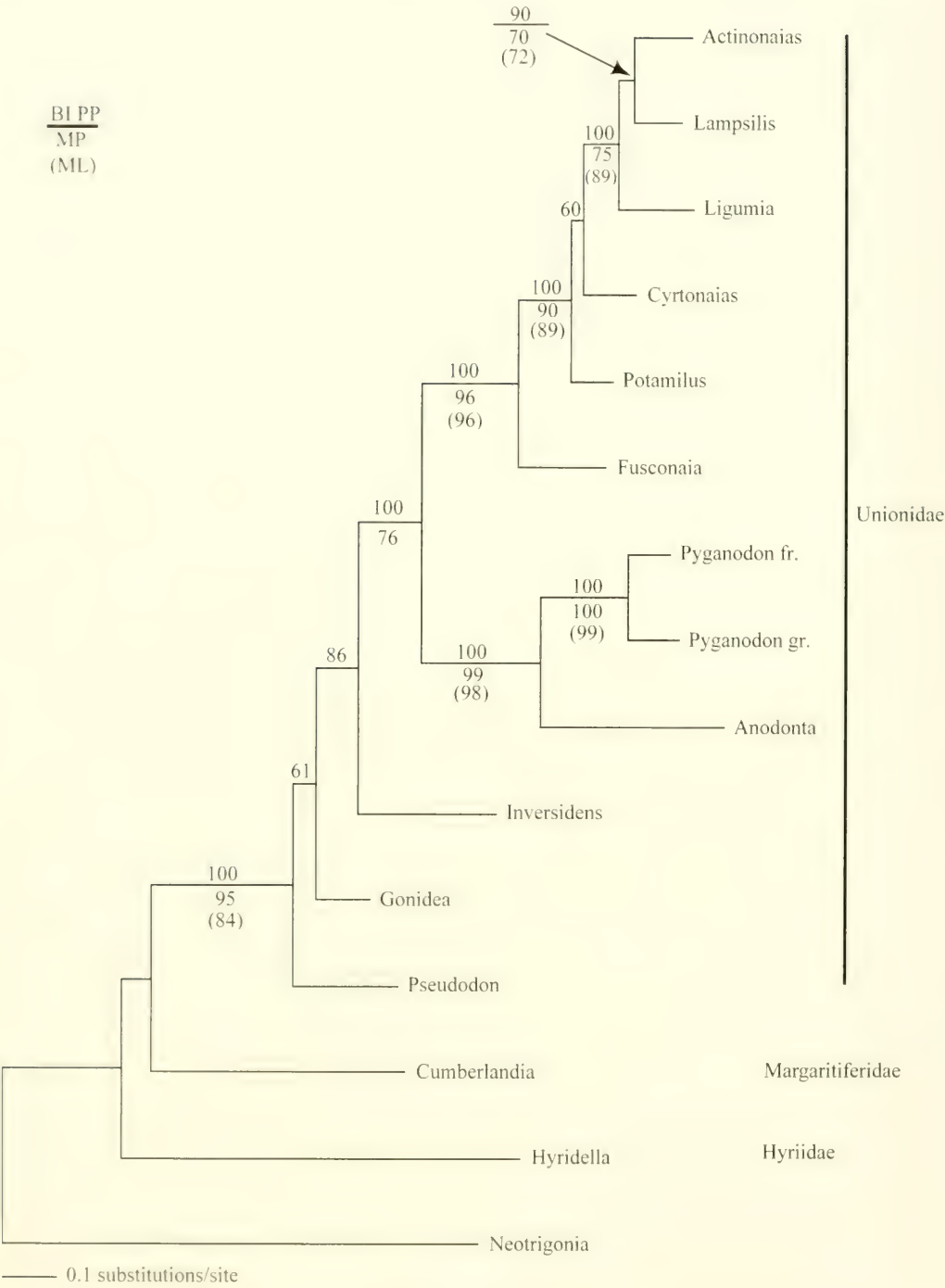


FIG. 3. Best tree found by Bayesian analyses of *M. cox1* sequences (619 bp). When > 50, Bayesian posterior probabilities (x100) presented above internodes, MP bootstrap values and ML bootstrap values (in parentheses) are presented below internodes.

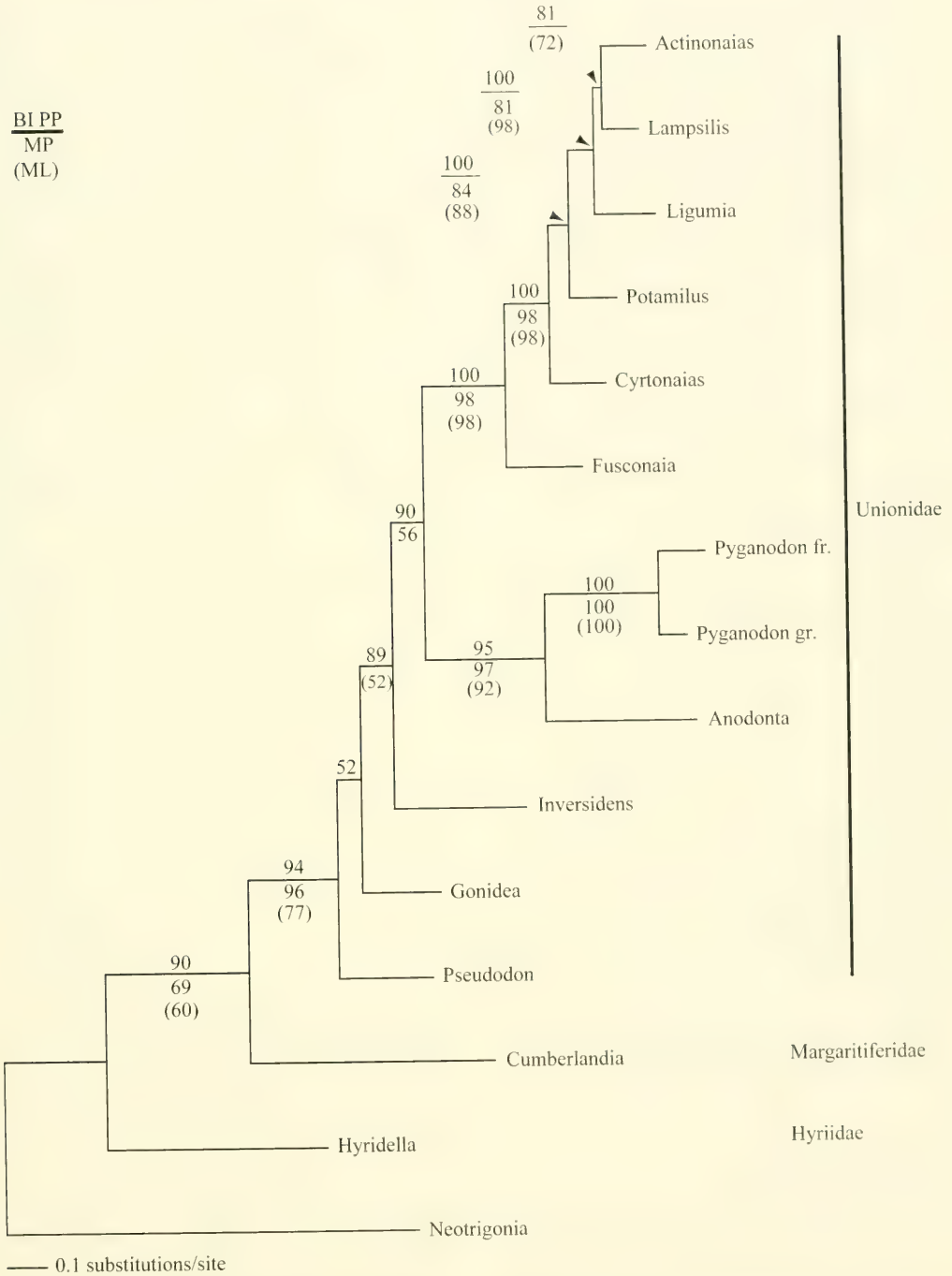


FIG. 4. Best tree found by Bayesian analyses of concatenated F + M *cox1* sequences (1238 bp). When > 50, Bayesian posterior probabilities (x100) presented above internodes, MP bootstrap values and ML bootstrap values (in parentheses) are presented below internodes.

higher level relationships are consistent with the relative relationships presented herein. One possibility is that the etherioidean lineage is the sister taxon to a monophyletic Unionoidea (Fig. 5A). This topology is consistent with the Parodiz & Bonetto (1963) unionoid classification. Deductions from this topology regarding DUI evolutionary dynamics are dependent on whether or not the outgroup, *Neotrigonia*, possesses DUI. If *Neotrigonia* lacks DUI, this topology would be consistent with the hypothesis of an ancestral unionoidean gain of DUI. Alternatively, if *Neotrigonia* possesses DUI, this

topology would be consistent with a loss of DUI in the ancestral etherioidean lineage. Under this topology, endobranchy is hypothesized as the ancestral unionoid brooding strategy but the two principal larval character states cannot be polarized. In addition, under this topology, the Parodiz & Bonetto (1963) "independent invasions of freshwater" hypothesis for unionoidean and etherioidean bivalves is not robustly rejected.

An alternative unionoid topology, that would maintain the relative evolutionary relationships represented in the trees from the complemen-

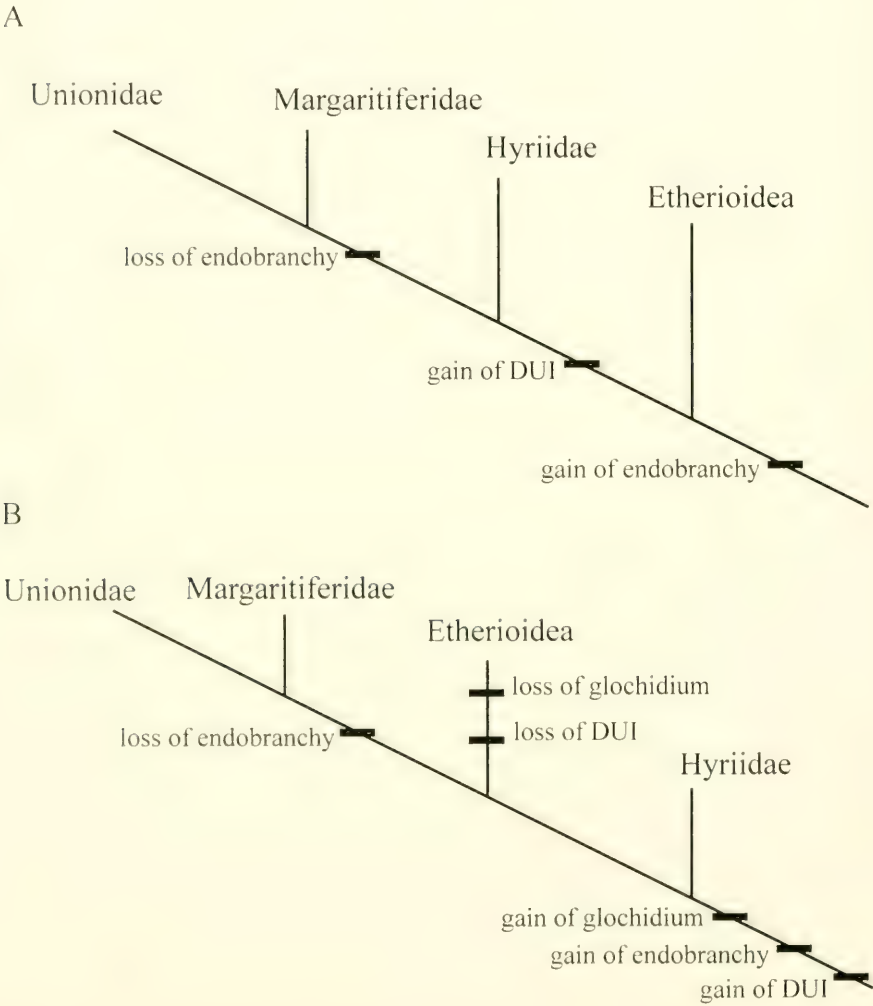


FIG. 5. Hypothesized character state transitions for reproductive characters exhibited by unionoid bivalves. A: Hypothesized familial relationships after Parodiz & Bonetto (1963); B: Hypothesized familial relationships after Hoeh et al. (2001). The two DUI character state optimizations displayed herein are based on the assumption that *Neotrigonia* lacks DUI.

tary analyses, is that the Etherioidea is sister taxon to the margaritiferid + unionid clade (Fig. 5B). This particular unionoid topology was supported by previous analyses using *F. cox1* sequences (Hoeh et al., 1998b, 2001). This topological placement would support the following hypotheses: (1) the ancestral etherioidean lineage lost DUI, (2) the glochidium is the ancestral larval type for the Unionoida, and (3) endobranchy is the ancestral brooding condition for the Unionoida. Additionally, this particular unionoid topology would reject the monophyly of the Unionoidea (*sensu* Parodiz & Bonetto, 1963) as well as Parodiz & Bonetto's "independent invasions of freshwater" hypothesis for unionoidean and etherioidean bivalves.

What Can Taxonomically Inclusive Analyses of Non-concatenated F and M *cox1* Sequences Tell us About Higher Level Unionoid Relationships?

The topology of the F genome portion of the inclusive BI analysis (Fig. 6) strongly supports the hypothesis that etherioids are the sister taxon to a margaritiferid + unionid clade (PP = 93), thus rendering the Unionoidea paraphyletic. This topology is congruent with the hypothesis of unionoid relationships presented in Figure 1C (after Hoeh et al., 1998b, 2001). It also strongly supports the monophyly of margaritiferid, mycetopodid, and etherioid bivalves (PP = 100 for each clade) and the paraphyly of the Unionidae (PP = 90). In contrast, monophyly of the Hyriidae is weakly supported (PP = 63) and iridinid monophyly was not supported. The results from the M-genome portion of the inclusive BI-based phylogenetic analysis of the *cox1* DNA sequences (Fig. 6) strongly support the sister taxa status (PP = 96) for the margaritiferid and unionid clades (PP = 100 for each family). Furthermore, both the F and M genome portions of this BI analysis strongly support the evolutionary propinquity of *Gonidea* and *Pseudodon* (PPs = 98 and 100, respectively). The topology obtained from the concatenated F and M *cox1* sequence analysis (Fig. 4) is in agreement with the former but not the latter hypothesis. In general, the MP bootstrap analysis of the inclusive non-concatenated *cox1* data set produced much lower nodal support values than did the BI analysis (Fig. 6).

The BI analysis presented in Figure 6 strongly supports the character state dynamics hypothesized in Figure 5B: (1) The presence of DUI, glochidial larvae, and endobranchous brooding characterized the ancestral unionoid lin-

eage, (2) the loss of DUI and glochidial larvae (i.e., the gain of standard maternal inheritance and lasidial larvae) occurred in the ancestral etherioidean lineage, and (3) the loss of endobranchy occurred in the ancestor of the margaritiferid + unionid clade. The hypothesis that the relatively "simple" anatomy of margaritiferids is a derived rather than an ancestral condition is also supported. Furthermore, the topology presented in Figure 6 strongly rejects the hypothesis that unionoidean and etherioidean bivalves represent independent invasions of freshwater habitat (i.e., unionoid bivalve polyphyly, as suggested by Parodiz & Bonetto, 1963). Reciprocal monophyly for the Etherioidea and Unionoidea, which would be consistent with the independent invasion hypothesis, is rejected by the topology presented in Figure 6.

At least two aspects of the phylogenetic results presented in Figure 6 should serve as a caution to any attempt to canonize these results: (1) only the BI analysis provided strong support for many of the higher level unionoid bivalve relationships discussed above and (2) the phylogeny for the Unionoida indicated in the F clade of Figure 6 is based on a relatively small number of nucleotides (a maximum of 619) from a single genetic locus (*F. cox1*). In order to more rigorously evaluate these central yet, in our opinion, currently open questions regarding unionoid higher level relationships and character state evolutionary dynamics, we are currently investigating the efficacy of incorporating DNA sequences from additional F mitochondrial genes (e.g., *F. cox2*). Including information from multiple female-transmitted unionoid mtDNA genes has the potential to increase the topological resolution of taxonomically inclusive analyses. In addition, nuclear genes (e.g., *28S*, *EF1-alpha*, *act42A*) as well as morphological characters are being investigated to assess their potential to facilitate the construction of a robust phylogeny for the Unionoida.

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